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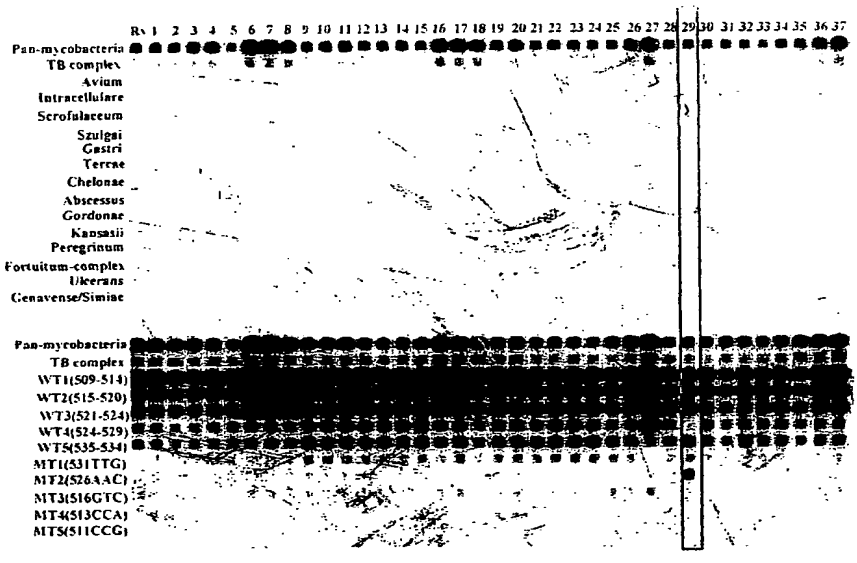
(54) Title: A METHOD FOR IDENTIFYING *MYCOBACTERIUM TUBERCULOSIS* AND NON-TUBERCULOSIS MYCOBACTERIUM, TOGETHER WITH DETECTING RESISTANCE TO AN ANTITUBERCULOSIS DRUG OF MYCOBACTERIUM OBTAINED BY MUTATION OF RPOB GENE



(57) Abstract: The present invention provides a method for identifying *Microbacterium tuberculosis (M.tuberculosis)* and Mycobacterium Other Than Tuberculosis (MOTT), and for determining the resistance to antj-tuberculosis drug obtained by mutation of M. tuberculosisand MOTT at the same time.

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A method for identifying *Mycobacterium tuberculosis* and non-tuberculosis Mycobacterium, together with detecting resistance to an antituberculosis drug of Mycobacterium obtained by mutation of *rpo*B gene

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a method for identifying *Mycobacterium tuberculosis* (Hereinafter, referred to as '*M. tuberculosis*') and Mycobacterium Other Than Tuberculosis (hereinafter, referred to as 'MOTT'), and at the same time for detecting the resistance obtained by mutation of *M. tuberculosis* and MOTT to an antituberculosis drug.

Description of the Related Arts

Tuberculosis is a chronic wasting disease caused by *M. tuberculosis*, which worldwide ranks the first in mortality and morbidity among infectious diseases (1) (Hereinafter, the number in the parentheses refers to the number in the below part of 'References'). Carriers of *M. tuberculosis* presently number about 1.9 billion, a third of the world population, and about 8～10 million of these carriers develop into new tuberculosis patients per year, and about 3 million of patients die of tuberculosis per year (2-4). Also, about a half of the population in Korea are carriers of *M. tuberculosis*, and about 150,000 persons develop into new tuberculosis patients per year, and about 14,000 patients of these die of tuberculosis per year (5).

Further, it was reported that the patients doubly infected with HIV virus and *M. tuberculosis* are much more susceptible to the tuberculosis, so that the seriousness of the

tuberculosis problem has become increasingly apparent, together with the increase of HIV fear by its rapid world-wide propagation. Presently, it is estimated that about 15 million patients are doubly infected with the *M. tuberculosis* and HIV, and also, most of these patients will probably develop into terminal tuberculosis patients (6).

In the meantime, although the tuberculosis should be continuously controlled to preserve the people's health in developing countries, insufficiency of antituberculosis drugs and improper treatment and control of tuberculosis in these countries result in the increase of patients with *M. tuberculosis* possessing the resistance to antituberculosis drugs (7). In particular, because of the appearance of *M. tuberculosis* possessing resistance to various antituberculosis drugs and the increase of the appearance frequency of such bacteria, the number of persons dying of tuberculosis has increased, and prompt elimination of tuberculosis has been obstructed (8-11). The treatments of such tuberculosis caused by *M. tuberculosis* possessing the resistance to antituberculosis drugs are quite costly, and the efficiency of its treatment is extremely low, thus the tuberculosis by *M. tuberculosis* with the resistance to antituberculosis drugs develops into incurable tuberculosis increasing the mortality from tuberculosis. Therefore, the propagation of such *M. tuberculosis* should be blocked by efficient treatment of the tuberculosis patients through its earlier diagnosis.

However, the drug-susceptibility test for *M. tuberculosis* used in almost all countries including Korea is performed by microbiological methods, which require long time periods of 8～10 weeks (12). Therefore, methods for rapidly and accurately determining the drug-susceptibility of *M. tuberculosis* are required, and it is considered that these methods may increase the efficiency of the tuberculosis treatment through providing proper treatment strategies. Ultimately these methods may, at an early stage, block the increase of incurable tuberculosis by the propagation of the drug-resistant *M.*

tuberculosis. Therefore, these methods are important health care techniques capable of reducing the national economic loss by prevention, elimination and treatment of tuberculosis.

Recently, methods for detecting the drug-resistance using genetic technologies have been developed for prompt drug-susceptibility tests. For example, it is reported that the mechanism by which *M. tuberculosis* obtains resistance to rifampin, one of the strongest effective antituberculosis drugs, results from nucleotide mutation in the 69bp nucleotides region of the gene encoding RNA polymerase(*rpo*B gene) β -subunit targeted by the above-mentioned drug (13, 14). At least, 97% of *M. tuberculosis* with resistance to rifampin obtains this resistance by the above mechanism. Therefore, kits for detecting *M. tuberculosis* with resistance to rifampin have been developed by the above mechanism.

The genus Mycobacterium includes *M. tuberculosis* causing tuberculosis, *M. lepraeae* causing Hansen's disease and other Mycobacterium species called Mycobacterium Other Than Tuberculosis (MOTT). MOTTs are bacteria causing opportunistic infections, and thus the MOTTs do not only infect patients who have reduced immunity, but also normal persons. Particularly, in the advanced countries after the year 1980, it was reported that MOTTs cause tuberculosis to those infected by HIV, thus the reports brought out the seriousness and importance of the MOTTs, together with the increase of AIDS patients. Further, the cases of diseases caused by various types of Mycobacteria have been increasingly found in advanced countries, therefore recently the rapid and accurate identification of Mycobacterial species has been recognized as being important.

Conventional methods for identifying *M. tuberculosis* and MOTTs have been performed to determine various microbiological and biochemical properties of

Mycobacterium species (15). However, such conventional identification methods require up to four weeks of growth time depending on the types of bacteria, thus the identification time is prolonged. In addition, the results obtained by the conventional methods are sometimes unclear, and some Mycobacterium species are not distinguished from other species by the conventional methods. Therefore, the results of identification are inaccurate. To overcome the above problems of the conventional identification method, the molecular biological methods for identification of MOTTs have been recently developed in the advanced countries. Particularly, the molecular biological methods for identifications of MOTTs using a gene including the nucleotide sequence region as the probe, which is highly conserved and changes while Mycobacterial species are evolving only among the same Mycobacterium species, were developed (16-19). Among these, the *rpo*B gene found by the present inventors may be used in the method for identifying Mycobacterium more simply, rapidly and accurately than the conventional molecular biological method using the nucleotide polymorphism of 16S rRNA (KR99-46795). That is to say, the Mycobacterial *rpo*B gene may be usefully employed for the preparation of the Mycobacterium species-specific primers, because the *rpo*B genes include highly conserved regions capable of being detected in all the species of Mycobacteria; also the *rpo*B genes from Entero-bacteriaceae other than Mycobacteria are not amplified by PCR, and if amplified, the sizes of PCR products are different from each other. Further, the results of the above prior studies by the present inventors show that the *rpo*B gene of Mycobacterium species includes nucleotide polymorphism regions, and thus, the *rpo*B gene may be used in the preparation of Mycobacterium species-specific probes for DNA-hybridization. Conclusively, the present inventors found in the prior studies that the 361bp region in the *rpo*B gene can be usefully utilized for the development of a method for identification of *M. tuberculosis* and MOTTs.

SUMMARY OF THE INVENTION

The purpose of the present invention is to provide a method for identifying *M. tuberculosis* and MOTTs, and at the same time for detecting resistance obtained by mutation of the *rpo*B gene to an antituberculosis drug of Mycobacterial species.

Another purpose of the present invention is to provide primers for PCR amplification of the *rpo*B gene used in identifying *M. tuberculosis* and MOTTs, and at the same time in detecting resistance obtained by mutation of the *rpo*B gene to an antituberculosis drug of Mycobacterial species.

Another purpose of the present invention is to provide oligomer probes and membranes adhered to by such oligomer probes for performing the reverse blot hybridization.

Still another purpose of the present invention is to provide a kit for identifying *M. tuberculosis* and MOTTs, and at the same time for detecting resistance obtained by mutation of the *rpo*B gene to an antituberculosis drug of Mycobacterial species, which comprises the said membranes adhered to by the said oligomer probes and the said primers or which comprises the said primers, the said oligomer probes and the said membranes.

DETAILED DESCRIPTION OF THE INVENTION

To achieve the above purposes, the present methods are characterized to identify *M. tuberculosis* and MOTTs, and to detect resistance obtained by mutation of the *rpo*B gene to an antituberculosis drug of Mycobacterium by the method comprising the following steps:

(1) isolating DNA from samples;

(2) amplifying the 531bp fragment in the *rpo*B gene by PCR using said DNA isolated in the above step (1) as template and MOTT-rpo-long-B-5' (5'-TCAAGGAGAAGCGCTACGACCTGGC-3'; SEQ. ID. NO. 1) and TR8-long-NB-3' (5'-ACGGGTGCACGTCGCGGACCTCCA-3'; SEQ. ID. NO. 2) as primers; and,

(3) performing PCR-reverse blot hybridization by hybridizing PCR products obtained in the above step (2) to the membranes adhered to by the oligomer probes of SEQ. ID. No. 3 to 30, wherein the oligomer probes of SEQ. ID. NO. 3 to 20 are species-specific oligomer probes binding to the corresponding Mycobacterial species and the oligomer probes of SEQ. ID. NO. 21 to 30 specifically bind to mutants of the *rpo*B gene conferring the drug resistance on *M. tuberculosis* or bind to the *rpo*B gene of the wild type.

The present invention is described in detail below.

In the above step (1), the samples are prepared from persons who are carriers or expected to be carriers of *M. tuberculosis*. At this time, it is preferable that such samples are obtained from patients' phlegm because *M. tuberculosis* is parasitic on their lungs. The isolation of DNA from said samples may be performed by methods commonly known to a person skilled in the art.

In the above step (2), said primers are used for PCR amplification of 531bp fragments in the *rpo*B gene illustrated in Fig. 1. As shown in Fig. 7, the 531bp fragment amplified by PCR, i.e. PCR product includes 1) the conserved sequence region which all Mycobacterium species possess, 2) the Mycobacterium species-specific polymorphism sequence region which each Mycobacterium possesses, and 3) the sequence region conferring the resistance by its mutation to antituberculosis drugs such as rifampin.

Therefore, the said amplified product of the *rpo*B gene may be used for identification of Mycobacterium, distinction between *M. tuberculosis* and MOTTs, identification of MOTTs and detection of resistance obtained by the mutation of *rpo*B gene to antituberculosis drugs of Mycobacterium.

Such a mutation in the rpoB gene renders *M. tuberculosis* resistant to antituberculosis drugs such as rifampin or its derivatives such as rifamycin and the like.

In the above step (3), the PCR products of the *rpo*B gene, which are obtained in the above step (2), are hybridized with the above oligomer probes by the reverse blot hybridization method. The sequences of oligomer probes which species-specifically hybridized with the corresponding Mycobacterium and specifically hybridized with wild types or mutant types of the *rpo*B region conferring resistance to antituberculosis drugs such as rifampin upon *M. tuberculosis* are shown in Table 1.

Table 1.

Name of Oligomer Probe	Sequence of Oligomer probe	Mycobacterium for targeting
MYC	GACGTCGTCGCCACCATCGA	All types of Mycobacteria
MTB	CATGTCGGCGAGCCC	*M. tuberculosis* complex
AVI	AAACGGTGAGCCGATCACC	*M. avium*
INT	AAACCTGCACGCGGGCGA	*M. intracellularae*
SCR	AAAAACGTACGGATGGCCAGC	*M. scrofulaceum*
KAN-I	AAAGGCCACGATGACCGTG	*M. kansasii* type I+V
KAN-II	AAAAATCTCAGGATGGCCAGC	*M. kansasii* type II+III+IV
GAS	AAAAATCTCAGGGTGGCCAGG	*M. gastri*
FOR-C	CCTGAACGCCGGCCAG	*M. fortuitum* complex
PER	GTTCCGGTCGAGGTGG	*M. peregrinum*
CHE	AAATGGTGACTGCCACCACG	*M. chelonae*
ABS	AAAAGGTGACCACCACCACC	*M. abscesus*
ULC	GGCCAGCCCATCACC	*M. ulcerans*
GEN/SIM	CCAGCCGACGATGACG	*M. genavanse / M. simiae*
GOR-I	AAAGTCGGCGATCCGATCA	*M. gordonae* type I, III, IV
GOR-II	AAAAACGTCGGCAAGCCGA	*M. gordonae* type II
SZU	AAATCTGAACGTCGGCGAG	*M. szulgai*
TER	AAAGCTCAGGACGGTCAGT	*M. terrae*
WT1	AACCAGCTGAGCCAATTC	Wild Type 509-514
WT2	ATGGACCAGAACAACCCG	Wild Type 515-520
WT3	AAACTGTCGGGGTTGACC	Wild Type 521-525
WT4	TTGACCCACAAGCGCCGA	Wild Type 524-529
WT5	CTGTCGGCGCTGGGGC	Wild Type 530-534

MT1	CTGTTGGCGCTGGGGC	Mutant Type 531TTG
MT2	AAAACCAACAAGCGCCGA	Mutant Type 526AAC
MT3	AATGGTCCAGAACAACCCG	Mutant Type 516GTC
MT4	AAAGCTGACCCCATTCAT	Mutant Type 513CCA
MT5	AAAGCCGAGCCCATTCAT	Mutant Type 511CCG

Each oligomer probe in the above Table 1 is constructed so as to species-specifically hybridize with the corresponding Mycobacterium, and whose sequences are described in the SEQ. ID. NO. 3 to 20.

The oligomer probes of WT1, WT2, WT3, WT4 and WT5 can specifically detect the wild type of nucleotides 509-514, 515-520, 521-524, 525-529 and 530-534 on the sequences of PCR products of *M.* In addition, oligomer probes of MT1, MT2, MT3, MT4 and MT5 can specifically detect TTG mutation on nucleotide 531, AAC mutation on nucleotide 526, GTC mutation on nucleotides 516, CCA mutation on nucleotides 513 and CCG mutation on nucleotides 511, respectively.

As shown in the Fig. 5, if the resulting band is not obtained when performing the reverse blot hybridization using the oligomer probes of WT1～5, the region coding the gene susceptible to rifampin is considered as being mutated. Therefore, the detection of said mutant types enables to determine whether *M. tuberculosis* has the resistance to rifampin or not.

It is preferable that the length of the oligomer probes, GC contents and position of improper mismatch in oligomer probes are modulated in order that the oligomer probes in Table 1 have high specificity and sensitivity to the corresponding Mycobacterium species, together with being hybridized at the same temperature. Especially, in case of oligomer probes having lower sensitivity, some non-specific nucleotides are added to their 5'-terminal in order to increase the efficiency of hybridizing the oligomer probes with the membrane and the corresponding target

specimen.

In addition, it is important that the oligomer probes are prevented from being moved freely on the membrane when the reverse blot hybridization according to the present invention is performed. To achieve this purpose, the amino groups are conjugated to 5'-terminal of the oligomer probes, and the resulting oligomer probes can be covalently linked with a carboxyl group on the membrane surface, resulting in the stable adhesion of the oligomer probes to the membrane. The membrane employed in the present invention is Biodyne-C membrane (Pall Biosupport, East Hills, NY), but the present invention is not limited to it, and any membranes may be preferably used if the carboxyl group exists on the membrane surface.

In short, the present invention provides primers used in amplifying the 531bp fragments in the *rpo*B gene by PCR, the oligomer probes described in the above Table 1 and the membranes adhered to by the said oligomer probes, in order to separately identify *M. tuberculosis* and MOTTs and detect resistance obtained by the mutation of *rpo*B gene to an antituberculosis drug of Mycobacterium.

Further, the present invention provides a kit comprising the said primers, the oligomer probes shown in Table 1 and the membranes, or comprising the said primers and the membranes adhered to by the oligomer probes shown in Table 1.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a genetic map of the *rpo*B gene from *M. tuberculosis*.

Figure 2 shows that the 531bp DNA fragments in the *rpo*B gene are amplified by PCR using DNA isolated from standard Mycobacterium species and the primers of SEQ. ID. NO. 1 and SEQ. ID. NO. 2.

(Wherein, M is Gene Ruler™ DNA size marker, lanes 1～13 are, respectively, standard species of *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. szulgai*, *M. gordonae*, *M. kansasii*, *M. abscessus*, *M. chelonae*, *M. gastri*, *M. fortuitum*, *M. ulcerans*, *M. terrae*, and lane 14 is negative control of PCR amplification.).

Figure 3 shows the results of the PCR reverse blot hybridization of the present invention for standard species of *M. tuberculosis* and MOTTs, wherein PCR products of each species are species-specifically hybridized with the corresponding species-specific oligomer probes.

Figure 4 shows the results of the PCR reverse blot hybridization of the present invention for the MOTTs isolated from clinical fields.

Figure 5 shows that the detection of the susceptibility or resistance to rifampin of *M. tuberculosis,* together with the identification of *M. tuberculosis* and MOTTs, are able to be performed by the PCR reverse blot hybridization according to the present invention (as shown in Fig. 5, lanes 1～6 represent *M. tuberculosis* with the susceptibility to rifampin; lane 7 represents *M. tuberculosis* with the resistance to rifampin due to the mutation of 514-520 region on PCR products from *M. tuberculosis*; lanes 8～15 represent *M. tuberculosis* with the resistance to rifampin due to the mutation of 524-529 region on PCR products; lane 16 represents *M. tuberculosis* with the resistance to rifampin due to the mutation of nucleotide 511 into CCG on PCR products; lane 17 represents *M. tuberculosis* with the resistance to rifampin due to the mutation of nucleotide 513 into CCA on PCR products; lanes 18～19 represent *M. tuberculosis* with the resistance to rifampin due to the mutation of nucleotide 516 into GTC on PCR products; lane 20 represents *M. tuberculosis* with the resistance to rifampin due to the mutation of nucleotide 526 into AAC on PCR products; lane 21 represents *M. tuberculosis* with the resistance to rifampin due to the mutation of nucleotide 531 into

TGC; and lanes 22~35 represent *M. tuberculosis* with the resistance to rifampin due to the mutation of nucleotide 531 into TTG on PCR products.).

Figure 6 shows the results in performing the detection of resistance to rifampin, at the same time the identification of *M. tuberculosis* and MOTTs by the PCR-blot hybridization for thirty-seven clinical samples.

Figure 7 shows the sequence of 531bp DNA fragment obtained by performing PCR amplification for *M. tuberculosis*.

In more detail, the present invention is explained by preferred embodiments and experimental examples. However, the present invention should not be limited to these examples, and it should be clearly understood that many variations and/or modifications of the basic inventive concepts herein taught which may appear to those skilled in the art will still fall within the spirit and scope of the present invention.

PREFERRED EMBODIMENTS

1. Mycobacterial species

Species described in Table 2 below are used in the present experiments as standard species of *M. tuberculosis* and MOTTs. The susceptibility test to rifampin of *M. tuberculosis* isolated from clinical fields was determined with the works of the Team for Detecting Drug Resistance of Tuberculosis in Molecular Biology Section, Department of Microbiology, the Korean Institute of Tuberculosis. Also, the identification of MOTTs was previously made using the microbiological and biochemical methods as the conventional methods by the Team for Identifying Mycobacterium Species in the above Molecular Biology Section, Department of Microbiology and the PCR-RFLP method

developed by the present inventors

Table 2

	Species	Strain	Source		Species	Strain	Source
1	*M. abscessus*	Pettenkofer Inst.	YUMC	23	*M. intermedium*	ATCC 51848	KIT
2	*M. africanum*	ATCC 25420	KIT	24	*M. kansasii* type I-V		Pasteur Inst.
3	*M. arcinogenes*	ATCC 35753	KIT	25	*M. malmoense*	ATCC 29571	KIT
4	*M. asiaticum*	ATCC 25276	KIT	26	*M. marinum*	ATCC 927	KIT
5	*M. aurum*	ATCC 23366	KIT	27	*M. microti*	ATCC 19422	KIT
6	*M. austroafricanum*	ATCC 33464	KRIBB	28	*M. moriokaense*	ATCC 43059	KRIBB
7	*M. avium*	ATCC 25291	KIT	29	*M. mucogenicum*	ATCC 49650	KIT
8	*M. bovis*	ATCC 19210	KIT	30	*M. neoaurum*	ATCC 25795	KIT
9	*M. bovis* BCG	French Strain 1173P2	KIT	31	*M. nonchromogenicum*	ATCC 19530	KIT
10	*M. celatum* type I/II	ATCC 51130/ ATCC 51131	KIT	32	*M. parafortuitum*	ATCC 19686	KIT
11	*M. chelonae*	ATCC 35749	KIT	33	*M. peregrinum*	ATCC 14467	KIT
12	*M. chitae*	ATCC 19627	KIT	34	*M. phlei*	ATCC 11758	KIT
13	*M. fallax*	ATCC 35219	KIT	35	*M. pulveris*	ATCC 35154	KRIBB
14	*M. fortuitum* type I/II	ATCC 6841/ ATCC 49404	KIT	36	*M. scrofulaceum*	ATCC 19981	KIT
15	*M. gallinarum*	ATCC 19710	KRIBB	37	*M. smegmatis*	ATCC 19420	KIT
16	*M. gastri*	ATCC 15754	KIT	38	*M. szulgai*	ATCC 35799	KIT
17	*M. genavense*	ATCC 51233	KIT	39	*M. terrae*	ATCC 15755	KIT
18	*M. gilvum*	ATCC 43909	KIT	40	*M. thermoresistibile*	ATCC 19527	KIT
19	*M. gordonae type I-IV*	ATCC 14470	KIT	41	*M. triviale*	ATCC 23292	KIT
20	*M. haemophilum*	ATCC 29548	KIT	42	*M. tuberculosis* H37Rv	ATCC 27294	KIT
21	*M. intracellulare*	ATCC 13950	KIT	43	*M. ulcerans*	ATCC 19423	KIT
22	*M. interjectum*	ATCC 51457	KIT	44	*M. vaccae*	ATCC 15483	KIT
				45	*M. xenopi*	ATCC 19250	KIT

2. Isolation of DNA

A loop of medium cultivating species described in the above Table 1 was suspended in 0.4 ㎖ of ddH_2O in 1.5 ㎖ experimental tube, and the bacteria solution was prepared by mixing the suspension. Then, through boiling the bacteria solution, the genomic DNA was obtained. Then, about 5 ㎕ of the above genomic DNA was used in PCR amplification.

In this experiment, DNA was isolated from patients' expectoration. That is to say, the expectoration was treated with 4% NaOH in the same amount of expectoration,

then this solution was poured into a 50 mℓ Felcon tube. Next, the solution was homogenized by vortexing, followed by reacting at room temperature for 15 minutes. Subsequently, distilled water was added to the reaction solution at a final volume of 50 mℓ, followed by centrifuging at 3,000rpm for 20 minutes. The supernatant was discarded and the pellet was vortexed, then 500 μℓ of the solution was poured into 1.5 mℓ tube. Then, 500 μℓ of 2% NaOH was added to the above tube, followed by mixing well using the vortex. Next, the solution was boiled for 2 minutes, and it was centrifuged at 12,000rpm for 3 minutes, followed by discarding the supernatant. The pellet was suspended in 1 mℓ of 0.1M Tris-Cl (pH 6.8). The suspension was vortexed, followed by centrifuging at 12,000rpm for 3 minutes, then the supernatant was discarded. The pellet, with 100 μℓ of glass bead solution added, was shaken for 1 minute and 30 seconds by Bead Beater. The DNA was obtained from the supernatant, and 5-10 μℓ of the DNA solution was used in the following PCR amplification.

3. PCR amplification

The PCR reaction mixture containing KCl 50mM, Tris-HCl 10mM (pH 8.3), $MgCl_2$ 1.5mM, gelatin 0.001% (w/v), dNTP each 200μ M, *Taq* polymerase 1.25 unit, primers 10pmol and genomic DNA 50-100ng at a final volume of 50 μℓ was prepared and used in PCR reaction. The PCR reaction was performed under the PCR conditions as follows: denaturation for 5 minutes at 95℃; a cycle of 30 seconds denaturation at 94℃, 30 seconds annealing at 58℃ and 45 seconds elongation at 72℃, repeated for 35 cycles; and final elongation reaction for 10 minutes at 72℃. The primer sequences employed in the above PCR amplification were as follows, and the region of PCR products amplified by the above PCR reaction is shown in Fig. 1. Further, the sequence of 531 bp DNA fragment amplified is shown in Fig. 7. :

MOTT-rpo-long-B-5' : 5'-TCAAGGAGAAGCGCTACGACCTGGC-3'

TR8-long-NB-3' : 5'-ACGGGTGCACGTCGCGGACCTCCA-3'

After completing the PCR amplification, the 531bp fragment of PCR product was confirmed by the electrophoresis of about 5 $\mu\ell$ of the reaction solution in 1.0% agarose gel.

4. oligomer probes

The probes for distinctly identifying Mycotuberculosis and specific MOTTs and the probes for detecting the susceptibility or the resistance of *M. tuberculosis* to rifampin were designed by using the corresponding *rpo*B gene, respectively. As each oligomer probe may be hybridized with the corresponding DNA at the same temperature, the length and the GC contents of each oligomer probe were adjusted so as to increase the specificity and the sensitivity to the corresponding DNA. In particular, in case of the oligomer probes having lower sensitivity, some non-specific nucleotides were added to their 5'-terminal so as to increase the efficiency with which the oligomer probe binds to the membrane and target samples. The sequences of each oligomer probe are described in Table 1. Further, the amino groups were conjugated to 5'-terminals of all the oligomer probes, and the amino groups of the oligomer probes were covalently bonded to the carboxyl group on the surface of the Biodyne-C membrane. The oligomer probes in the membrane for the reverse blot hybridization were covalently bonded to the above Biodyne-C membrane, thereby the membrane for performing the reverse blot hybridization was prepared.

5. PCR-reverse blot hybridization

After the PCR amplification, PCR-reverse blot hybridization was performed

using 10 ㎕ of the 531bp PCR product confirmed by the electrophoresis in agarose gel and the Biodyne-C membrane adhered to by the oligomer probes prepared in the above. The oligomer probes and PCR products were loaded using Miniblotter-MN45 (Immunetics, Cambridge, MA).

In brief, PCR-reverse blot hybridization was performed as follows. The amplified and confirmed PCR product 10 ㎕ was diluted by adding 150 ㎕ of 2x SSPE/0.1% SDS solution. Next, the diluted PCR product was dissociated at 99℃ for 10 minutes, followed by cooling in ice. Before loading PCR product on the membrane, the membrane was soaked in 100 ㎖ of 2x SSPE/0.1% SDS solution and stored at room temperature for 5 minutes. Then, this membrane was positioned on the support cushion in the Miniblotter. After removing remaining moisture in the slot by aspirator, the diluted PCR product was loaded in the slot, wherein the slot was vertically positioned in the direction of the adhering oligomer probes. The direction of the adhering oligomer probe was previously indicated on the membrane using ink. The empty slot around the slot loaded PCR product was filled with 2x SSPE/0.1% SDS in order to prevent the cross-flow. The hybridization reaction was performed in 50℃ flat-ground incubator for 2 hours. For preventing the cross-flows from slots to near slots, the membrane was not shaken. Afterwards the sample was removed from the Miniblotter using an aspirator and the membrane was also removed from the Miniblotter, then the membrane was washed two times with 100 ㎖ of 2xSSPE/ 0.5% SDS solution at 57℃ for 10 minutes. The membrane was then put in a rolling bottle, and 10 ㎖ of streptavidine-alkaline phosphatase conjugate solution diluted with 2x SSPE/0.5% SDS solution by 1:2000 was poured into the membrane, followed by reacting at 42℃ for 60 minutes. The membrane was then washed two times with 100 ㎖ of 2x SSPE/0.5% SDS solution at 42℃ for 10 minutes, and two times with 100 ㎖ of 2x SSPE solution for 5 minutes.

After the hybridization was completed, the above membrane was treated with 10 ㎖ of CDP-Star™ detection reagent (Amersham pharmacia biotech., Buckinghamshire, England) for 4 minutes for the chemiluminiscent detection. Next, the membrane was wrapped with overhead sheet or wrap and exposed to X-ray film for 30 minutes (if needed, can be further exposed to X-ray film up to 2 hours), and the experimental results are obtained.

6. Results

1) PCR amplification of *rpo*B gene by using Mycobacterium standard species

The *rpo*B gene regions were amplified from 54 kinds of the standard species (furnished from ATCC) including subtypes kept in the Korean Institute of Tuberculosis in the same manner as above. The results are shown in Fig. 2. As shown in Fig. 2, in case of using the primers MOTT-rpo-long-B-5' and TR8-long-NB-3', 531bp DNA fragments including the region for separately identifying *M. tuberculosis* and MOTTs and the region related to the resistance to antituberculosis drugs were amplified, irrespective of species type (*see* Fig. 1).

2) PCR-reverse blot hybridization by using *rpo*B gene from Mycobacterium standard species

The PCR products amplified by PCR using the genomic DNA from Mycobacterium standard species were hybridized with the membranes adhered to by species-specific oligomer probes, thereby it was confirmed that each Mycobacterium species may be identified separately. The result is shown in Fig. 3. As shown in Fig. 3, (1) most of the Mycobacterial species bound to Mycobacterial species-specific oligomer

probes, (2) the only PCR product from the corresponding Mycobacterium species hybridizes with the species-specific oligomer probes. These results indicate that the oligomer probes according to the present invention are very useful in separate identification between *M. tuberculosis* and MOTTs. Moreover, it was confirmed that the species-specific oligomer probes are not hybridized with the PCR product from other Mycobacterium species except for the corresponding Mycobacterium species.

As shown in Fig. 3, in case of *M. gordonae*, *M. kansasii* and *M. fortuitum*, some subtypes belonging to the one species exist, and some of sequence polymorphism were formed in these subtypes, and therefore the present invention designed and used the oligomer probes that may be hybridized with all subtypes in each species. Although, *M. terrae* did not hybridize with the oligomer probe that targets all Mycobacterium, it was specifically hybridized with the oligomer probe specific to *M. terrae*, thus the identification of *M. terrae* was of no difficulty. In addition, although *M. genavense* has a tendency to hybridize with the oligomer probe specific to *M. tuberculosis*, it can be also hybridized specifically with the oligomer probe specific to *M. genavense*, thus the identification of *M. genavense* was of no difficulty.

3) PCR-reverse blot hybridization by using *rpo*B gene from Clinical Mycobacterium species isolated from Korean Institute of Tuberculosis

The identification of clinical Mycobacterium species was performed by the PCR-reverse blot hybridization from clinical Mycobacterium species in the same manner as above. For the above experiment, *M. tuberculosis* previously identified by the Team for Identifying Mycobacterium Species in the Korean Institute of Tuberculosis, by microbiological, biochemical and molecular biological methods such as the PCR-RFLP method and MOTTs with clinical meanings or with high appearance frequency were used

as the clinical Mycobacterium species. Examples of MOTTs with clinical meanings or with high appearance frequency include the pathological MOTTs such as *M. avium-intacellulare* complex, *M. kansasii*, *M. marinum*, *M. fortuitum*, *M. chelonae*, *M. abscessus*; pathogenic MOTTs with less appearance frequency such as *M. malmoense*, *M. asiaticum*, *M. xenopi*, *M. simiae*, *M. scroflaceum*, *M. nonchromogenicum*, *M. peregrinum*, *M. szulgai*, *M. haemophilum*, *M. ulcerans*; and, non-pathogen MOTTs with high appearance frequency such *M. terrae*, *M. gordonae*. Among these, MOTTs generally found in the Korean Institute of Tuberculosis include *M. avium*, *M. intracellulare*, *M. fortuitum*, *M. terrae*, *M. gordonae*, *M. chelonae*, *M. abscessus* and the like.

Therefore, among the above MOTTs with clinical meanings or with high appearance frequency, the PCR-reverse blot hybridization was performed for MOTTs species with high appearance frequency in Korea. MOTT species applied to this experiment and the results are shown in Fig. 4. As shown in Fig. 4, each clinical Mycobacterium species analyzed through this experiment was specifically hybridized with the corresponding oligomer probes. As a result, it is confirmed that the result of the identification by the method of the present invention is equal to that by previous microbiological, biochemical and molecular biological methods.

Therefore, the method of the present invention enables the identification of Mycobacterium species rapidly and highly sensitively. Further, it is confirmed that various species of MOTTs coexist in mixed populations. This result may not be obtained with previous microbiological methods and biochemical analyses, and the PCR-RFLP methods.

4) Identification of *M. tuberculosis*, together with determination of the susceptibility to rifampin

In order to confirm that the susceptibility to rifampin of *M. tuberculosis* may be determined by the method of the present invention, the PCR amplification of the *rpo*B gene and the PCR-reverse blot hybridization were performed in the same manner as above. The result is shown in Fig. 5. As shown in Fig. 5, it was confirmed that the discrimination between rifampin-resistance and rifampin-susceptibility of *M. tuberculosis* was detected exactly, while both Mycobacterial groups were identified as *M. tuberculosis*.

5) Identification of Mycobacterium and determination of drug susceptibility to rifampin using DNA isolated from expectoration samples

DNAs were isolated from thirty-seven samples of phlegm of patients diagnosed as smear positive by acid fast staining (32), and the *rpo*B gene region was amplified by PCR using the above isolated DNAs, followed by performing PCR-reverse blot hybridization using the amplified PCR product in the same manner as above. Thereby, the identification of Mycobacterium species, together with determination of the susceptibility to rifampin was performed. As a result, it was confirmed that the bacteria in smear positive expectoration samples were identified as *M. tuberculosis* and MOTTs separately, and the bacteria identified as *M. tuberculosis* were divided into rifampin-susceptible and rifampin-resistant tuberculosis distinctly at the same time. The result is shown in Fig. 6. As shown in Fig. 6, *M. tuberculosis* was detected in all samples, in particular the sample in lane 29 was determined to be rifampin-resistant bacteria.

As described in the above, the method of the present invention may separately identify *M. tuberculosis* and MOTTs by the use of the PCT products of the *rpo*B gene. And in case of identification as tuberculosis bacteria, the method of the present invention may at the same time detect its resistance obtained by mutation of the *rpo*B to

antituberculosis drug such as rifampin. Therefore, the present invention may reduce the time required for bacteria identification and drug resistance, as a total of 1～2 weeks is required for identification of bacteria and detection of resistance to antituberculosis drugs, while the previous microbiological method requires a total of 3～4 weeks for the identification and the detection, respectively. Also, the results obtained by performing the present invention are very accurate. In addition, identification of Mycobacterium and determination of the susceptibility to rifampin are easily performed by persons other than those skilled in the art because the opportunity for contact with bacteria is very low.

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CLAIMS

1. A method for identifying *Mycobacterium tuberculosis* and non-tuberculosis Mycobacterium (MOTT), and at the same time for detecting the resistance or the susceptibility of *M. tuberculosis* obtained by mutation of the *rpo*B gene to antituberculosis drug, which comprises the following steps:

(1) isolating DNA from a sample;

(2) amplifying the 531bp fragment in the *rpo*B gene by PCR using said DNA isolated in the above step (1) and the primers of SEQ. ID. NO. 1 and SEQ. ID. NO. 2;

(3) performing PCR-reverse blot hybridization by hybridizing the PCR products obtained in the above step (2) with the membranes adhered to by the oligomer probes of SEQ. ID. No. 3 to 30.

2. The method according to claim 1, wherein said antituberculosis drug is rifampin or its derivatives.

3. The primers comprising sequences described in SEQ. ID. NO. 1 and SEQ. ID. NO. 2, which are used in amplifying the 531bp fragment in the *rpo*B gene by PCR.

4. The oligomer probes comprising sequences described in SEQ. ID. NO. 3 to 30, which are used in identifying Mycobacterium tuberculosis and MOTTs and at the same time in detecting their resistance or susceptibility obtained by mutation of the *rpo*B gene to antituberculosis drug.

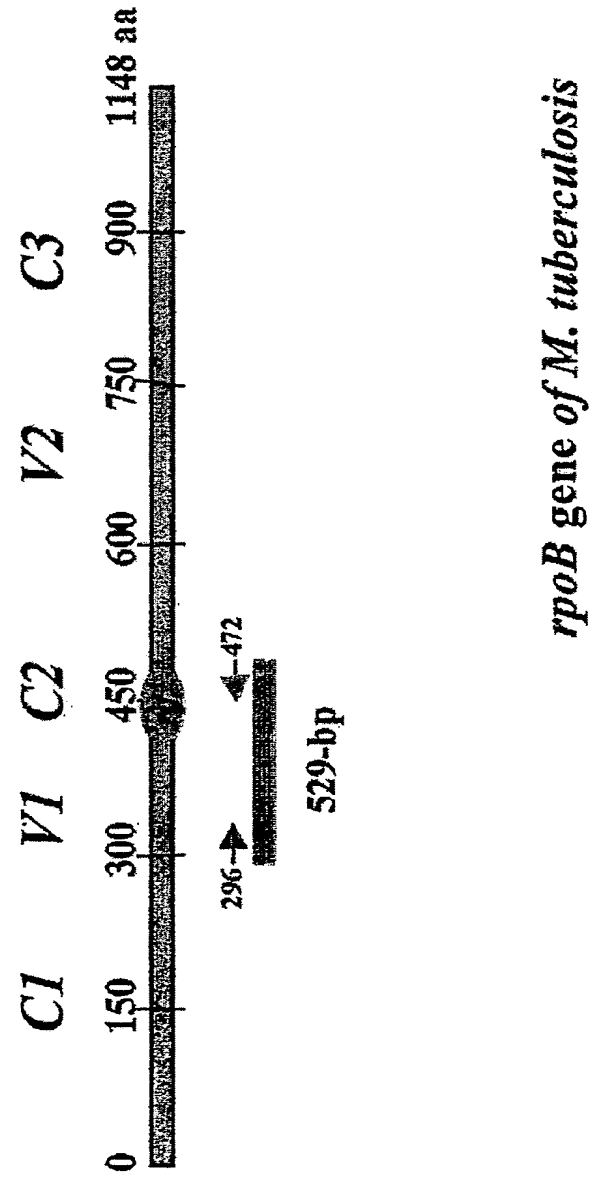
5. The membranes adhered to by the oligomer probes of claim 4.

6. The membranes according to claim 5, whereby said membranes are Biodyne-C membranes.

7. A kit for identifying Mycobacterium tuberculosis and MOTTs and at the same time for detecting their resistance or susceptibility obtained by mutation of the *rpo*B gene to antituberculosis drug, which comprises the primers described in claim 3, the oligomer probes described in claim 4 and the membranes described in claim 5.

Figure

FIG. 1


C1
V1
C2
V2
C3
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296
472
529-bp
rpoB gene of M. tuberculosis

FIG. 2

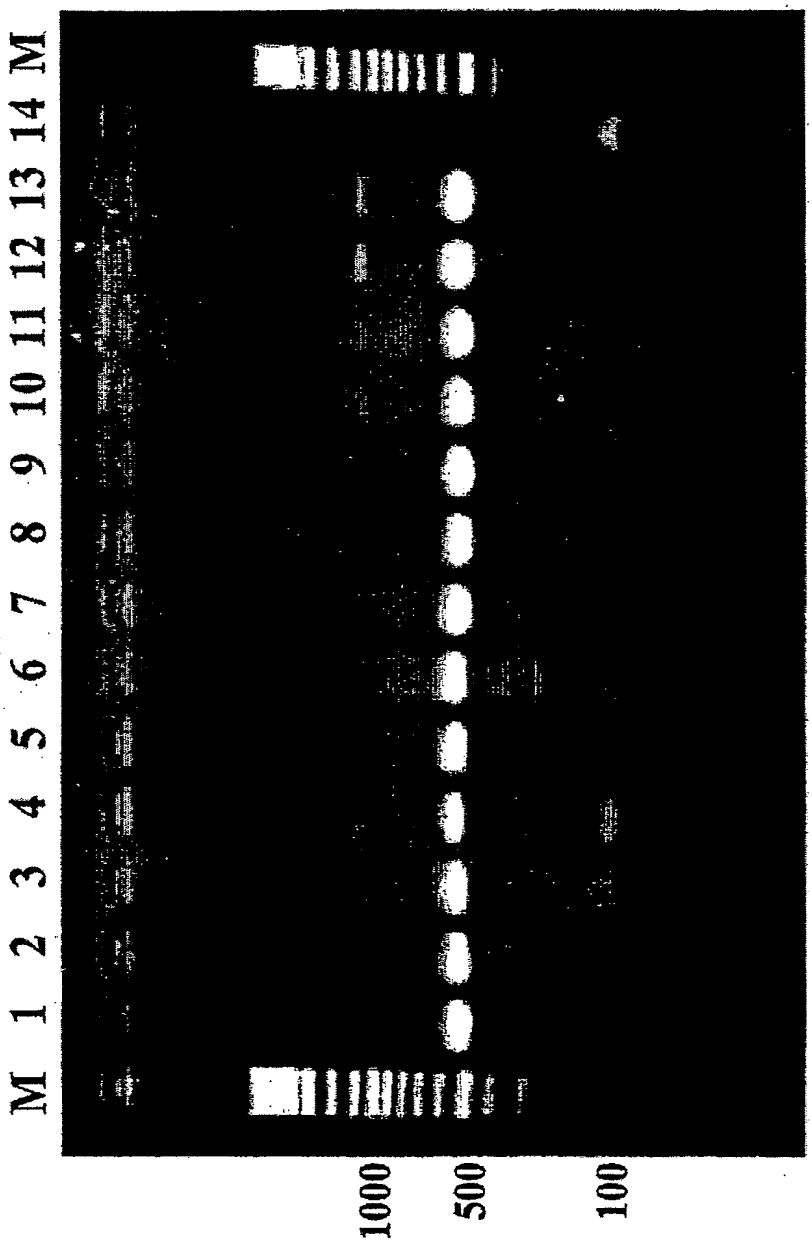
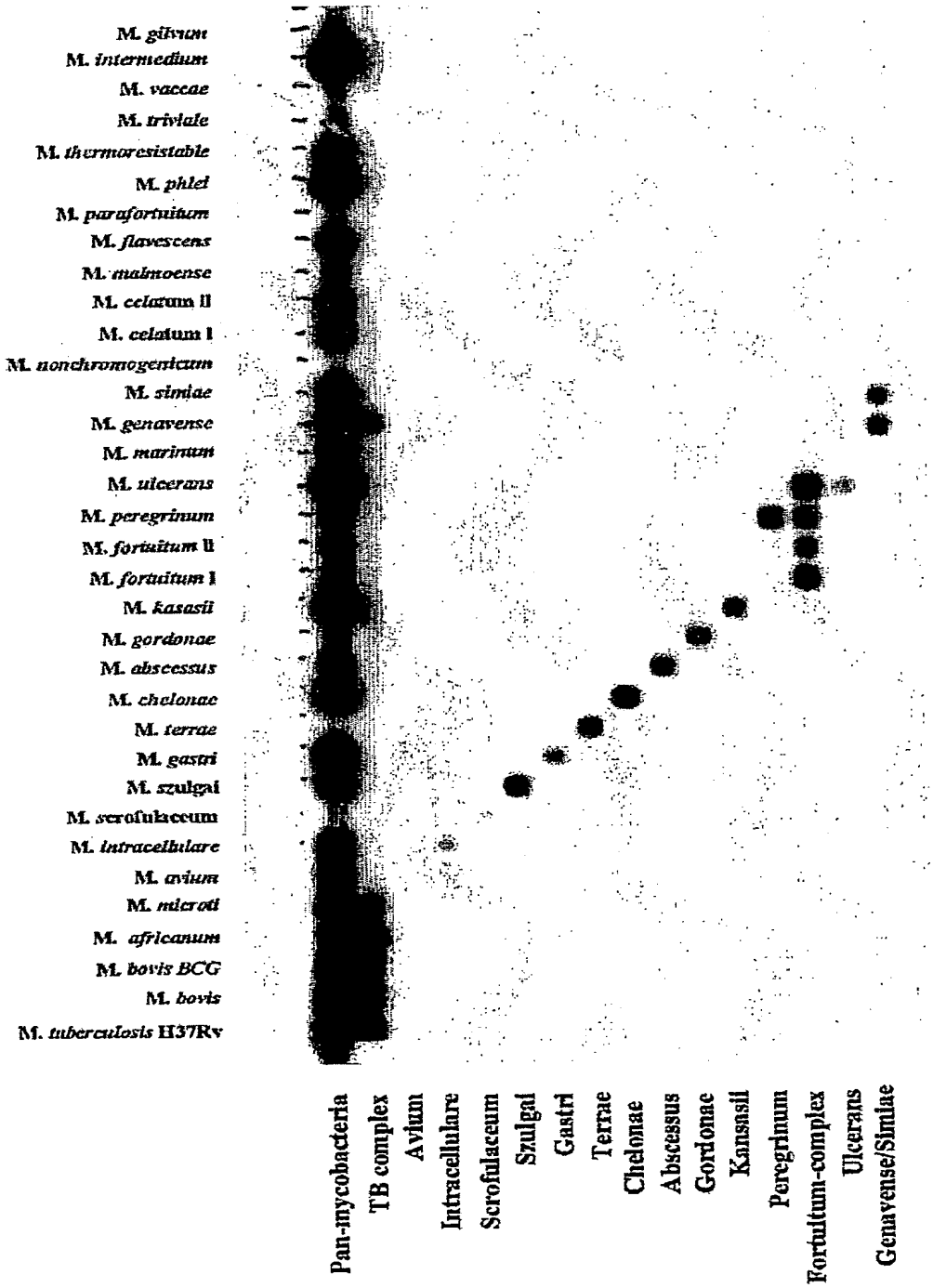


FIG. 3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M. intermedium
M. vaccae
M. triviale
M. thermoresistable
M. phlei
M. parafortuitum
M. flavescens
M. malmoense
M. celatum II
M. celatum I
M. nonchromogenicum
M. simiae
M. genavense
M. marinum
M. ulcerans
M. peregrinum
M. fortuitum II
M. fortuitum I
M. kasasii
M. gordonae
M. abscessus
M. chelonae
M. terrae
M. gastri
M. szulgai
M. scrofulaceum
M. intracellulare
M. avium
M. microti
M. africanum
M. bovis BCG
M. bovis
M. tuberculosis H37Rv
Pan-mycobacteria
TB complex
Avium
Intracellulare
Scrofulaceum
Szulgai
Gastri
Terrae
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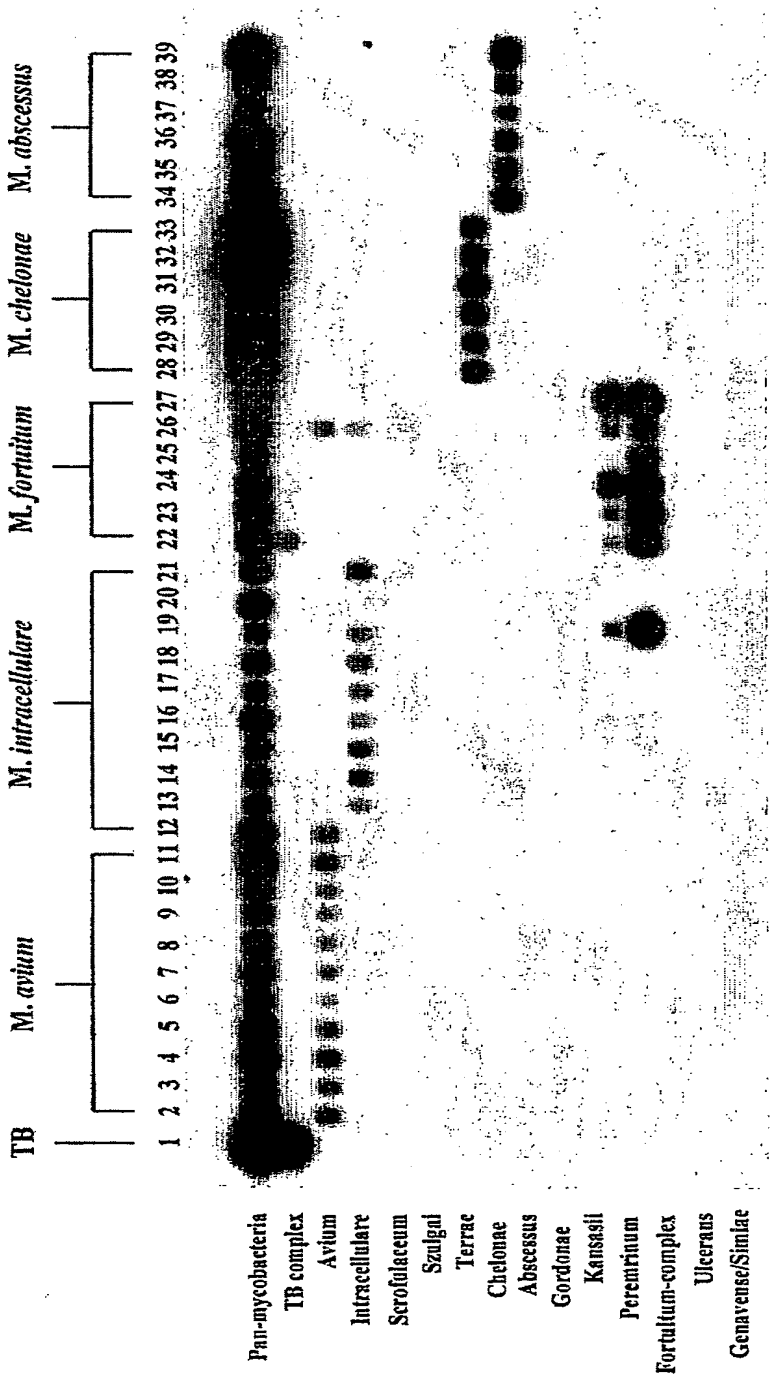
Abscessus
Gordonae
Kansasii
Peregrinum
Fortuitum-complex
Ulcerans
Genavense/Similae

FIG. 4


TB
M. avium
M. intracellulare
M. fortuitum
M. chelonae
M. abscessus
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Pan-mycobacteria
TB complex
Avium
Intracellulare
Scrofulaceum
Szulgai
Terrae
Chelonae
Abscessus
Gordonae
Kansasii
Peremtrium
Fortuitum-complex
Ulcerans
Genavense/Similae

FIG. 5


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
Pan-mycobacteria
TB complex
Avium
Intracellulare
Scrofulaceum
Szulgai
Gastri
Terrae
Chelonae
Abscessus
Gordonae
Kansasii
Peregrinum
Fortuitum-complex
Ulcerans
Genavense/Simiae
Pan-mycobacteria
TB complex
WT1(509-514)
WT2(515-520)
WT3(521-524)
WT4(525-529)
WT5(530-534)
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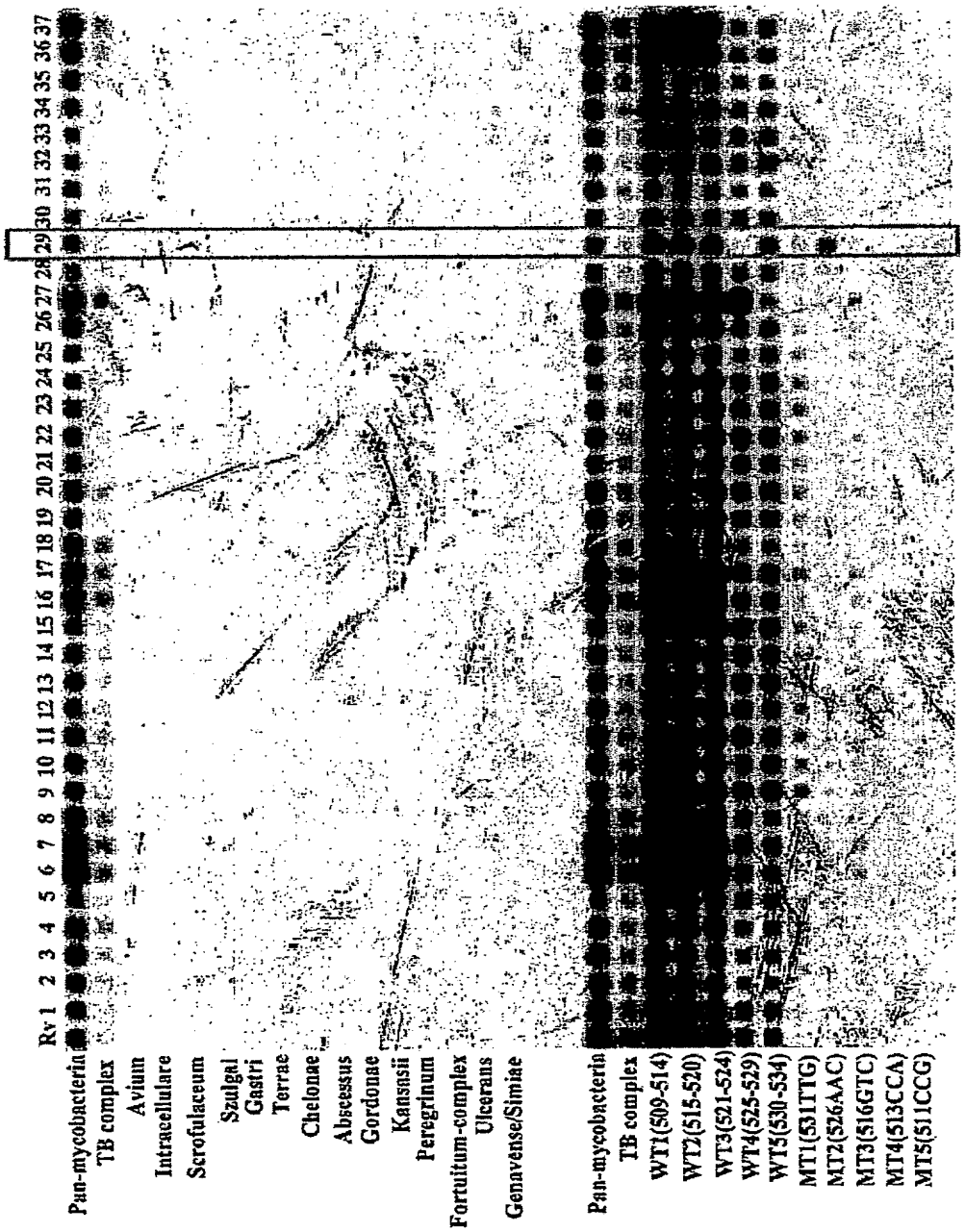
MT5(511CTG)

FIG. 6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Pan-mycobacteria
TB complex
Avium
Intracellulare
Scrofulaceum
Szulgai
Gastri
Terrae
Chelonae
Abscessus
Gordonae
Kansasii
Peregrinum
Fortuitum-complex
Ulcerans
Genavense/Simiae
Pan-mycobacteria
TB complex
WT1(509-514)
WT2(515-520)
WT3(521-524)
WT4(525-529)
WT5(530-534)
MT1(531TTG)
MT2(526AAC)
MT3(516GTC)
MT4(513CCA)
MT5(511CCG)

FIG. 7

Total: 531 bp

tca aggagaagcg ctacgacctg gcccgcgtcg gtcgctataa (43)

ggtcaacaag aagctcgggc tgcatgtcgg cgagcccatc acgtcgtcga cgctgaccga (103)

agaagacgtc gtggccacca tcgaatatct ggtccgcttg cacgagggtc agaccacgat (163)

gaccgttccg ggcggcgtcg aggtgccggt ggaaaccgac gacatcgacc acttcggcaa (223)

ccgccgcctg cgtacggtcg gcgagctgat ccaaaaccag atccgggtcg gcatgtcgcg (283)

gatggagcgg gtggtccggg agcggatgac cacccaggac gtggaggcga tcacaccgca (343)

gacgttgatc aacatccggc cggtggtcgc cgcgatcaag gagttcttcg gcaccagtcg (403)

gctgagccaa ttccatgcgc agaacaaccc gctgtcgggg ctgaccgaca agcgccgact (463)

gtcggccgcg gggccggcg gtctgtcacg tgagcgtgcc gggctgagag tccgcgacgt (523)

gcacccgt

Underlined Letters: Primer-binding regions

Bold Letters: polymorphic region existing in *M.tuberculosis* and MOTTs

Italic letters: the region related to the resistance against rifampin

<110> Xeniss Life Science Co., LTD.

<120> A method for identifying Micobacteria tuberculosis and non-tuberculosis Micobacteria, together with detecting resistance to an antituberculosis drug of Micobacteria obtained by mutation of rpoB gene

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/01253

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline, PAJ "Mycobacterium and rpoB and resistance and primer"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, A	WO 0192573 A1 (KIM, HYUN-JUNG et al.) 06 DECEMBER 01	1-7
A	US 6,242,584 B1 (KOOK, YOON-HOH et al.) 05 JUN 01	1-7
A	KR 01-38701 A1 (LEE, HYEYOUNG et al.) 15 MAY 01 (cited in the text)	1-7

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevence

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 APRIL 2002 (29.04.2002)	29 APRIL 2002 (29.04.2002)

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR01/01253

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0192573 A1	06.12.01	AU 6276701	
KR 01-38701 A1	15.05.01	none	
US 6,242,584 B1	05.06.01	AU8464898	
		KR97-65731 A1	13.10.97